

[ SEQ CHAPTER \h \r 1]**Primary** Chris Wozniak, Ph.D., Biotechnology Special Assistant, **Date:**  
**Reviewer:** \_\_\_\_\_

[ SEQ CHAPTER \h \r 1]**Secondary** [ SEQ CHAPTER \h \r 1]  
**Reviewer:** Jeannette Martinez, Ph.D., Emerging Technologies Branch **Date:** \_\_\_\_\_

### DATA EVALUATION RECORD

[ SEQ CHAPTER \h \r 1]**REQUIREMENT:** EPA OCSPP 850.4100

**TEST MATERIAL (PURITY):** *Aedes aegypti* OX513A

**SYNONYMS:** OX513A

**CITATION:**

Tetracycline-Repressible Transactivator Protein Variant (tTAV) and Related Genetic Material from OX513A *Aedes aegypti*: ANALYSIS OF NO EFFECT TO THREATENED OR ENDANGERED SPECIES OR CRITICAL HABITAT, MRID 504435-11

**SPONSOR:** Oxitec Ltd, 71, Milton Park, Abingdon, Oxfordshire, OX14 4RX  
United Kingdom

**AUTHOR:** Oxitec Ltd.

**TEST SITE:** Not applicable

**CITATION:** Volume 15, EUP Submission; MRID 504435-14, November 29, 2017

**COMPLIANCE:** Good Laboratory Practice Standards, 40 CFR Part 160, are not applicable to this document. This study is a request to waive the data generation requirements for an analysis of potential threats to endangered and threatened species study.

**This DER does not contain FIFRA CBI.**

**EXECUTIVE SUMMARY:**

The developer of the male-sterile *Aedes aegypti* OX513A mosquito, Oxitec Ltd., requests an analysis of their finding of no impact to threatened or endangered species or critical habitat (United States Endangered Species Act (ESA) (16 U.S.C. §§ 1531-1544, or Federally designated

critical habitat) following release of these mosquitoes. Oxitec has indicated that the male-sterile Yellow Fever mosquito, *Ae. aegypti* OX513A, will not result in adverse effects to threatened and endangered species based on: 1) should the OX513A mosquito be ingested by a listed species, no adverse effect is expected because neither the tTAV (tetracycline repressible transactivator variant) nor the DsRed2 (modified *Discosoma* red fluorescent protein) are toxins. Second, OX513A *Aedes aegypti* is an urban or domestic mosquito closely associated with human habitations. Non-target organisms in these areas are not usually threatened or endangered species. Third, there are no species that are obligate consumers of *Aedes aegypti* mosquitoes or for which *Aedes aegypti* mosquitoes comprise a significant proportion of the diet. Fourth, upon completion of the proposed trial, the population of *Aedes aegypti* is expected to be restored to its pre-field trial population level. Fifth, *Aedes aegypti* is a non-native species in the U.S. and has therefore, not co-evolved with other organisms in the ecosystem.

### **PURPOSE OF THE STUDY:**

To examine the potential interactions between threatened and endangered species and the male-sterile *Ae. aegypti* OX513A, as well as any influence on critical habitat utilized by threatened and endangered species of all types.

### **CLASSIFICATION: ACCEPTABLE**

#### **I. Description of Product**

**Strain/Source:** OX513A was developed through microinjection of the tTAV and DsRed2 genetic material into *Aedes aegypti* mosquito eggs of the Rockefeller strain. The insert was then introgressed into an *Aedes aegypti* Latin strain background from Instituto Nacional de Salud Publica (INSP), Mexico. The resulting OX513A line has been maintained in a continuously cycling insect colony. Sterile males, homozygous for the two transgenes, are to be released for population suppression; a very low number of homozygous (tTAV, DsRed2) females may be inadvertently included following mechanical separation of pupae prior to rearing adult mosquitoes for release.

The OX513A mosquitoes express the tTAV protein constitutively, however, in the presence of tetracycline, the tTAV protein preferentially binds the tetracycline molecule rather than the tetracycline response element (tRE), inhibiting gene expression. The presence of tetracycline, therefore, prevents the overexpression of tRE genes and subsequent transcriptional squelching, which ultimately leads to cell death in the absence of tetracycline or its analogues. Death of OX513A in the absence of tetracycline typically occurs in the late larval or pupal stages of the *Ae. aegypti* life cycle (Phuc et al., 2007).

DsRed2 is a modified red fluorescent protein derived from a coral-like anemone (Anthozoan) of the genus *Discosoma*. The presence of this RFP allows for identification of the OX513A larvae and pupae and distinction from wild type *Ae. aegypti*.

Following rearing in the presence of tetracycline, the larvae pupate and size differences between male and female pupae allow for physical separation. Approximately 99.8% of adults obtained following separation of pupae are male and 0.2% or less are female.

#### **Function of Product and Mode of Action**

The OX513A male-sterile *Ae. aegypti* released into identified receiving environments mate with wild-type *Ae. aegypti* resulting in egg and larval production, however, the presence of the tTAV transgene prohibits the morphogenesis of larvae into adult mosquitoes in the absence of tetracycline. The tTAV protein binds to the tetO DNA sequence in the absence of tetracycline or its derivatives (e.g., oxytetracycline, doxycycline) and enhances expression of this tetracycline responsive element (tRE). This overexpression results in a phenomenon known as transcriptional silencing (Gill and Ptashne, 1988) when expressed intracellularly, however, it does not represent toxicity to cells via direct contact or ingestion as this protein does not contain toxic moieties. The expression of tTAV at high levels in a positive feedback loop reduces the production of other critical proteins necessary for cellular maintenance (Lin et al., 2007; Ant et al., 2012). Expression of the tTAV transgene is expected to result in death of approximately 95% of OX513a larvae. Should the OX513A mosquitoes be consumed or contacted by threatened or endangered species, no adverse effect will occur and any impacts on critical habitat will be the same as for wild-type *Ae. aegypti*.

The OX513A release is intended to reduce, but not eliminate, local *Ae. aegypti* populations. As is similar with chemical (e.g., organophosphate, pyrethroid) or biological (e.g., *Bacillus thuringiensis* var. *israelensis*) mosquito abatement treatments, the decrease in mosquito populations is temporary and populations will rebound from unaffected mosquitoes or gradual movement into the treated area over time. Released male-sterile *Ae. aegypti* applied to a treatment area are anticipated to move less than 200 meters as they are weak fliers and tend to remain primarily in the area of release (LaCroix et al., 2012). Wind storms and occasional animal vectors may transport a small portion of these released mosquitoes outside the immediate treatment areas. Given the short timespan in which these modified insects remain viable (i.e., 2 to 3 days average), they are not expected to spread significantly or establish.

The DsRed2 transgene, which is included in OX513A as a visually detectable marker protein, is not known to be toxic via direct contact or ingestion via consumption. Its presence in the environment will not present a risk to threatened or endangered species nor negatively impact the habitat of such species. Based upon bioinformatic analyses, neither DsRed2 or tTAV are known to share significant sequence homology with known toxins or allergens (Goodman, 2017). Both of these proteins are susceptible to common proteases based upon bioinformatics analysis and digestion in simulated gastric and intestinal assays. These proteins are not expected to persist in the environment nor in the tissues of any animals ingesting mosquitoes containing these proteins.

#### **Impacts on Animals from Release of OX513A**

The OX513A *Ae. aegypti* releases will contain > 99.8 % male mosquitoes and therefore will not constitute a significant hazard related to female mosquitoes biting animals or transmitting disease, such as those caused by arboviruses. The OX513A laboratory colony is evaluated for the presence of several different arboviruses and lots would be rejected if the presence of any of these was confirmed.

As noted above, *Ae. aegypti* is predominantly a peridomestic resident and focuses primarily on human hosts when seeking a bloodmeal; hence, any females inadvertently released would be unlikely to interact with threatened or endangered animal species. The urban nature of this species and its preference for humans as a source of a bloodmeal make interactions with threatened or endangered species far less likely than with many other mosquito species.

Various species of bats, birds, fish, reptiles, amphibians, and insects are known to consume adult or larval mosquitoes with the species of mosquitoes ingested varying by habitat, seasonality and prey preferences. Adult *Ae. aegypti* frequent urban environments predominantly, hence their availability as food for many predatory species will be reduced based upon proximity to human dwellings and the lack of or reduced presence of such predators in the immediate area. Similarly, *Ae. aegypti* preferentially oviposit in clean water associated with various containers (e.g., tires, flower pots, gutters, cisterns) which are commonly associated with human habitation. Hence, consumption of larval *Ae. aegypti* by fish and amphibians is unlikely in such habitat. A study submitted (MRID# 503264-01) in support of this experimental use permit demonstrated a lack of toxicity or adverse effects to guppies (*Poecilia reticulata*) fed OX513A larvae (MRID# 563264-01). Predatory mosquito species *Toxorhynchites splendens* and *T. amboinensis* similarly consumed OX513A larvae without any evidence of adverse effects (Nordin et al., 2013: MRID# 504435-07).

Chiropteran species are considered as active generalist predators of insects and it has been anecdotally suggested that insectivorous bats may consume 1000 or more mosquitoes per hour or approximately 12,000 per night. This suggestion stems in part from extrapolations of a study (Griffin et al., 1960) conducted in a sealed environment wherein mosquitoes were the only prey made available to captive bats. The intent of the study was to evaluate echolocation characteristics of *Myotis* spp. in finding *Culex quinquefasciatus*, the southern house mosquito, on the wing, not to establish the bat's dietary preferences. In areas where larger, more nutritious insect prey are available, bats do not consume large numbers of mosquitoes as they do not constitute significant calories or nutrients relative to the task of predating upon them (Gonsalves et al., 2013; Wetzler and Boyles, 2018). While northern bats (*Myotis septentrionalis*) readily consume mosquitoes in enclosures, but evidence suggests that they consume few mosquitoes in an open feeding environment (Boyles et al., 2013).

A study of Big Brown Bats' (*Eptesicus fuscus*) prey preferences, a generalist feeder, indicated a predominance of Coleoptera, Diptera, Ephemeroptera and Lepidoptera species in their diet with Dipteran species predominated by chironomids and very few mosquitoes (Clare et al., 2014). Similarly, a comparison of the diet of eight bat species in southern Illinois concluded that mosquitoes represented a small portion of the overall diet of these insectivorous bats (Feldhamer et al., 2009).

Under certain conditions, such as colder nights where larger insects were less available or when female bats are lactating, Diptera, including mosquitoes and crane flies, may constitute a larger portion of the diet of the southeastern brown bat, *Myotis austroriparius*, in Florida (Zinn and Humphrey, 1983). These Dipterans constituted as much as 75% of biomass sampled by bats on cooler nights, however, the diversity of the diet of this insectivorous bat increased considerably

during warmer temperatures (*i.e.*, most spring and summer nights). In a recent Wisconsin study, little brown bats (*Myotis lucifugus*) and big brown bats (*Eptesicus fuscus*) were found to include mosquitoes (9 species identified) in their diet at 72% and 33% of samples, respectively, at all sites sampled (Wray et al., 2018). In contrast, Whitaker and Lawhead (1992) found mosquitoes in 17% of fecal samples of *M. lucifugus* which constituted 1.8% (volumetrically) of their insect-based diet. Given the taxonomic, temporal and geographical breadth of the studies referenced above, it can be concluded that for different insectivorous bat species, mosquitoes may constitute less or more of their overall dietary intake of insects depending in part on seasonality, bat species and availability of diverse prey.

Numerous insectivorous avian species include mosquitoes within their diet, however, none are known to include a significant biomass of mosquitoes as a regular part of their consumed prey. Purple Martins (*Progne subis*) have been considered as actively seeking mosquitoes among other flying insects when feeding. A study in Oklahoma evaluating prey captured by Purple Martins at various altitudes failed to detect mosquitoes as part of prey fed to young birds (Helms et al., 2016). Other reports suggest that mosquitoes typically do not make up more than 3% of the Purple Martin diet (Miller, 2006).

In a seven-year study conducted in Edinboro, PA, mosquitoes were not detected in 500 Purple Martin diet samples collected and analyzed (PMCA, 2006). Based upon the feeding habits of Purple Martins, it was considered as unlikely that the birds and freshwater mosquitoes would encounter each other routinely.

In contrast, Western Bluebirds, *Sialia mexicana*, consumed *Aedes* (species not identified) as the most common arthropod prey among 66 species identified in fecal samples (Jedlicka et al., 2017) from California vineyards. Samples from adults and nestlings indicated 51% and 49%, respectively, contained *Aedes* as prey.

Reptiles and amphibians are known to predate on mosquitoes, both adult and larval stages. While neither group has evolved to specifically target mosquitoes as a major portion of their diet, in some instances, mosquitoes can constitute a significant source of prey. The Tiger Salamander, *Ambystoma tigrinum*, was found to readily consume mosquito (Culicidae) larvae based on 26% of stomach samples containing remnants of larvae (Brodman and Dorton, 2006). Interestingly, salamander length was negatively correlated with the number of mosquito larvae consumed but positively correlated with consumption of larger prey (e.g., tadpoles, beetles). Mosquito larvae were determined to be the third most commonly consumed prey in this pond study. Eastern Spotted Newts, *Notophthalmus viridescens*, were also found to consume mosquito larvae among other insect prey in aquatic situations (Matheson and Hinman, 1929).

In addition to direct consumption, mosquito larvae and amphibian larvae (*i.e.*, tadpoles) may directly compete for resources and impact growth of each other, as well as survival (Mokany and Shine, 2003). While most tadpoles are herbivorous, some species will consume mosquito larvae and other insects in certain situations. It has been suggested that some mosquitoes will preferentially oviposit in waters with few or no tadpoles present.

**BPPD Comments:**

*Aedes aegypti* is known to frequent households and associated habitat in close proximity to buildings inhabited by humans. Adult male mosquitoes of *Ae. aegypti*, and many other mosquito species, are known to obtain nectar from various plant species as a source of sugar, vitamins and energy. Interactions with higher plants is minimal for mosquitoes and there is no plausible mechanism for toxicity of *Ae. aegypti* or the transgenes it expresses, tTAV and DsRed2, toward a wide range of species via consumption or casual contact.

The analysis request is appropriate considering the minimal exposure to threatened and endangered species and their critical habitat from release of OX513A male mosquitoes. A lack of plausible mechanisms of toxicity for these two transgenes following ingestion or direct contact with the OX513A mosquitoes indicates no adverse impacts from such exposure.

**CONCLUSION:** Acceptable

**References:**

Boyles, J.G., Catherine L. Sole, C.L., Cryan, P.M., and G.F. McCracken (2013) On Estimating the Economic Value of Insectivorous Bats: Prospects and Priorities for Biologists, *In*, R.A. Adams and S.C. Pedersen (eds.), *Bat Evolution, Ecology, and Conservation*, DOI 10.1007/978-1-4614-7397-8\_24, © Springer Science+Business Media New York

Brodman, R. and Dorton, R. (2006) The Effectiveness of Pond-Breeding Salamanders as Agents of Larval Mosquito Control, *Journal of Freshwater Ecology*, 21:3, 467-474.  
DOI: 10.1080/02705060.2006.9665024

Canyon D.V. and Hii, J.L. (1997) The gecko: an environmentally friendly biological agent for mosquito control. *Med Vet Entomol.* Oct;11(4):319-23

Clare, E.L., Symondson, W.O.C., and Fenton, M.B. (2014) An inordinate fondness for beetles? Variation in seasonal dietary preferences of night-roosting big brown bats (*Eptesicus fuscus*). *Molecular Ecology* (2014) 23, 3633–3647

Feldhamer, G.A., Carter, T.C., and Whitaker, JR, J.O. (2009) Prey Consumed by Eight Species of Insectivorous Bats from Southern Illinois. *Am. Midl. Nat.* 162:43-51

Gill, G., Ptashne M. (1988) Negative effect of the transcriptional activator GAL4. *Nature* 334, 721-724. August 25, 1988.

Gonsalves, L., Bicknell, B., Law, B., Webb, C., Monamy, V. (2013) Mosquito Consumption by Insectivorous Bats: Does Size Matter? PLoS ONE 8(10): e77183.  
doi:10.1371/journal.pone.0077183

Goodman RE. (2017) Updated bioinformatics analysis for risks of allergenicity and toxicity of proteins encoded by the two genes introduced into genetically engineered mosquitos (*Aedes aegypti*), strain OX513A for production of sterile males to reduce vector transmission of important human diseases. Unpublished report prepared by Food Allergy Research and Resource Program. Food Science and Technology. University of Nebraska. Study Number: REG Oxitec OX513A\_2. January 8, 2017. 132 p. EPA MRID 50326403.

Griffin, D.R., Webster, F.A., and Michael, C.R. (1960) Echolocation of flying insects by bats. *Animal Behaviour* 8(3-4):141-154.

Lacroix, R., McKemey, A.R., Raduan, N., Wee, L.K., Ming, W.H., Ney, T.G., Siti Rahidah A.A., Salman, S., Selvi Subramaniam, Oreenaiza Nordin, Norhaida Hanum A.T., Chandru Angamuthu, Suria Marlina Mansor, Rosemary S. Lees, Neil Naish, Sarah Scaife, Pam Gray, Genevieve Labbe', Camilla Beech, Derric Nimmo, Luke Alphey, Seshadri S. Vasan, Lee Han Lim, Nazni Wasi A., Shahnaz Murad (2012) Open Field Release of Genetically Engineered Sterile Male *Aedes aegypti* in Malaysia. PLoS One 7(8): e42771.  
doi:10.1371/journal.pone.0042771

Lin H., McGrath J., Wang P., Lee T. (2007) Cellular toxicity induced by SRF-mediated transcriptional squelching. *Toxicol Sci* 96: 83-91.

Matheson, R. and E.H. Hinman. 1929. The vermilion spotted newt (*Diemictylus viridescens*) as an agent in mosquito control. *Am. J. Hygiene* 9: 188-191.

Mokany, A. and R. Shine (2003) Competition between tadpoles and mosquito larvae. *Oecologia* 135:615-620

Nordin O., Donald W., Ming W., Ney T., Mohamed K., Halim N., Winskill P., Hadi A., Muhammad Z., Lacroix R. et al., (2013) Oral Ingestion of Transgenic RIDL *Ae. aegypti* Larvae Has No Negative Effect on Two Predator *Toxorhynchites* Species. *PLoS ONE* 8: e58805  
doi:10.1371/journal.pone.0058805. (Volume 8 of the EUP application).

Phuc, H.K., Andreasen, M.H., Burton, R.S., Vass, C., Epton, M.J., Pape, G., Fu, G., Condon, K.C., Scaife, S., Donnelly, C.A., Coleman, P.G., White-Cooper, H. and Alphey, L. (2007) Late-acting dominant lethal genetic systems and mosquito control. *BMC Biology* 5:11  
doi:10.1186/1741-7007-5-11 [ HYPERLINK  
"https://bmcbiol.biomedcentral.com/articles/10.1186/1741-7007-5-11" ]

Purple Martin Conservation Association (PMCA) (2006) The Purple Martin Conservation Association in cooperation with Edinboro University of Pennsylvania.  
<http://www.purplemartin.org>

USDA-FS, United States Department of Agriculture, Forest Service and Department of Interior, Bureau of Land Management (2009) Species Fact Sheet – Purple Martin, Updated May 2009, by Rob Huff, Oregon State Office, BLM [ [HYPERLINK "https://studylib.net/doc/7750091/species-fact-sheet---usda-forest-service"](https://studylib.net/doc/7750091/species-fact-sheet---usda-forest-service) ] [accessed 11-20-18]

Wetzler, G.C. and Boyles, J.G. (2018) The energetics of mosquito feeding by insectivorous bats. Canadian Journal of Zoology, 2018, Vol. 96 (4):373-377; [ [HYPERLINK "https://doi.org/10.1139/cjz-2017-0162"](https://doi.org/10.1139/cjz-2017-0162) ]

Whitaker, J. O., Jr., and B. Lawhead (1992) Foods of *Myotis lucifugus* in a maternity colony in central Alaska. Journal of Mammalogy 73: 646–648.

Wray AK, Jusino, M.A., Banik, M.T., Palmer, J.M., Kaarakka, H., White, J.P., Lindner, D.L., Gratton, C., and Peery, M.Z. (2018) Incidence and taxonomic richness of mosquitoes in the diets of little brown and big brown bats. Journal of Mammalogy 99(3):668-674. [ [HYPERLINK "https://doi.org/10.1093/jmammal/gyy044"](https://doi.org/10.1093/jmammal/gyy044) ]

Zinn, T.L. and S.H. Humphrey (1983) Seasonal food resources and prey selection of the southeastern brown bat (*Myotis austroriparius*) in Florida. Florida Scientist 44:81-90.